# High farnesoid X receptor expression predicts favorable clinical outcomes in PD-L1$^{low/negative}$ non-small cell lung cancer patients receiving anti-PD-1-based chemo-immunotherapy

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**Abstract.** Anti-programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1)-directed immunotherapy has revolutionized the treatment of advanced non-small cell lung cancer (NSCLC). However, predictive biomarkers are still lacking, particularly in identifying PD-L1$^{low/negative}$ patients who will benefit from immunotherapy. It was previously reported that farnesoid X receptor (FXR) downregulated PD-L1 expression in NSCLC, and that FXR$^{high}$PD-L1$^{low}$ mouse Lewis lung carcinoma tumors showed an increased susceptibility to PD-1 blockade compared with mock tumors. At present, whether the FXR$^{high}$PD-L1$^{low}$ phenotype predicts clinical response to immunotherapy in patients with NSCLC remains unclear. Herein, a retrospective study was conducted to examine the expression levels of FXR, PD-L1 and CD8$^+$ T cells by immunohistochemistry in a cohort of 149 patients with NSCLC receiving anti-PD-1-based chemo-immunotherapy. The results revealed that high FXR and PD-L1 expression levels were associated with higher objective response rates (ORR) in all patients. High PD-L1 expression also indicated superior progression-free survival (PFS). Interestingly, an inverse correlation was identified between FXR and PD-L1 expression in specimens with NSCLC. Subgroup analysis revealed that high FXR expression was associated with a higher ORR, as well as longer PFS and overall survival (OS) in PD-L1$^{low}$ patients. Cox multivariate analysis revealed that high FXR expression was an independent predictor for PFS and OS in PD-L1$^{low}$ patients. Tumor microenvironment evaluation revealed a statistically significant decrease of infiltrating CD8$^+$ T cells in FXR$^{high}$ specimens with NSCLC. Overall, the present study proposed an FXR$^{high}$PD-L1$^{low}$ signature as a candidate predictor of response to anti-PD-1-based chemo-immunotherapy in PD-L1$^{low/negative}$ patients with NSCLC, providing evidence that could be used to broaden the patients benefitting from immunotherapy.

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*Abbreviations:* NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; PD-1, programmed death-1; IHC, immunohistochemistry; TMB, tumor mutational burden; TME, tumor microenvironment; FXR, farnesoid X receptor; BA, bile acid; LLC, Lewis lung carcinoma; RECIST 1.1, response evaluation criteria in solid tumors version 1.1; PFS, progression-free survival; OS, overall survival; FFPE, formalin-fixed paraffin-embedded; S-W, Shapiro-Wilk; K-W, Kruskal-Wallis; ORR, objective response rate; HR, hazard ratio; CI, confidence interval

*Key words:* farnesoid X receptor, predictor, anti-PD-1-based chemo-immunotherapy, non-small cell lung cancer, PD-L1$^{low/negative}$ patients

## Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of all diagnosed lung cancers, and is the leading cause of cancer-related mortality worldwide with an estimated 1.8 million deaths in 2020 (1,2). Despite recent advances in surgery, radiotherapy, chemotherapy and targeted therapy, the prognosis of NSCLC remains dismal and the 5-year survival rate is lower than 20% (3). Programmed death-ligand 1 (PD-L1) or programmed death-1 (PD-1) blockade immunotherapy has resulted in striking clinical benefits in NSCLC, since nivolumab and pembrolizumab have been approved as first- or second-line treatments for advanced NSCLC, either

as monotherapy or in combination with chemotherapy (4-7). However, due to the primary or acquired resistance and adverse effects, only a small fraction of patients with NSCLC can benefit from immune-related therapies (8-10).

Tumor PD-L1 expression detected by immunohistochemistry (IHC) is the only approved biomarker for predicting response to anti-PD-1/PD-L1 immunotherapy (11). Several clinical trials have demonstrated superior overall survival (OS) for PD-1/PD-L1 blockade in NSCLC patients with high PD-L1 expression, compared with those with low PD-L1 expression (12,13). Alternative predictive biomarkers, such as tumor mutational burden and the tumor microenvironment (TME), have also been intensively investigated; however, conclusive evidence is lacking (14,15). It is noteworthy that the predictive value of PD-L1 expression is affected by multiple variables, including the different testing platforms and cut-off criteria for positivity, intra-tumoral and inter-tumoral heterogeneity and the dynamic change of PD-L1 expression (16-18). In fact, clinical efficacy was also observed in patients with cancer among the PD-L1$^{low/negative}$ group (6,19), suggesting that tumor PD-L1 expression alone is insufficient to recognize patients sensitive to PD-1/PD-L1 blockade. Future studies may help develop new predictors, particularly in identifying potential responding candidates to anti-PD-1/PD-L1 among the PD-L1$^{low/negative}$ patients.

Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that is predominantly expressed in the liver and gastrointestinal tract (20). As a bile acid (BA)-activated transcription factor, FXR regulates the expression of target genes involved in BA homeostasis, lipid and glucose metabolism (21,22). Previous studies have demonstrated the important role of FXR, either as an oncogene or as a tumor-suppressive gene, in the tumorigenesis of liver, colorectal, esophageal and breast cancer (23-26). It was previously reported that FXR is upregulated in NSCLC, compared with pericarcinous lung tissues and that FXR contributes to NSCLC cell proliferation via transactivating *CCND1* (27). In a recent study, an inhibitory role of FXR in PD-L1 expression in NSCLC was identified (28). Critically, FXR$^{high}$PD-L1$^{low}$ mouse Lewis lung carcinoma (LLC) tumors were more vulnerable to anti-PD-1 therapy than mock LLC tumors (28). However, whether or not FXR$^{high}$PD-L1$^{low}$ phenotype predicts clinical response to immune-related therapies in clinical patients with NSCLC has yet to be investigated. The present study aimed to determine the predictive value of FXR for anti-PD-1-based chemo-immunotherapy in the setting of clinical NSCLC, mainly focusing on the PD-L1$^{low/negative}$ group. In addition, the potential correlation between FXR expression and tumor-infiltrating CD8$^+$ T cells was also revealed.

## Materials and methods

*Patients and data collection.* From January 2019 to April 2021, 149 patients (119 men and 30 women; median age, 64; range, 38 to 75 years) with pathologically confirmed NSCLC who were scheduled to receive anti-PD-1-based chemo-immunotherapy at Shandong Provincial Hospital (Jinan, China) were screened. Certain patients who relapsed after complete resection were also included in the cohort, and their tumor-node-metastasis (TNM) staging information was determined at the beginning of anti-PD-1-based chemo-immunotherapy. Individuals who were aged 18-75 years, had pathologically confirmed stage III-IV NSCLC (according to the 8th edition of the AJCC/UICC classification for NSCLC) and were ineligible for radical surgery or radiotherapy, had at least one measurable lesion per the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1) (29), had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) (30) no more than 2, and had archival tumor tissues obtained within 6 months before chemo-immunotherapy or fresh tumor samples, were included. Those who had symptomatic central nervous system metastasis, had used immunosuppressants within 2 weeks before chemo-immunotherapy, had received neoadjuvant chemotherapy, radiotherapy or immunotherapy, or had a history of other malignant tumors were excluded. Patients were administered intravenous anti-PD-1 agents, including camrelizumab, sintilimab, tislelizumab, pembrolizumab (at a dose of 200 mg, every 3 weeks) and toripalimab (at a dose of 240 mg, every 3 weeks), combined with chemotherapies such as cisplatin, carboplatin, pemetrexed, gemcitabine and paclitaxel according to the standards of relevant guidelines. Clinical and pathological information, including age, sex, smoking history, histologic type, TNM stage, ECOG PS and lines of therapy, were retrospectively obtained from the medical records.

The present study was approved (approval no. NSFC-2019-05) by the Institutional Review Board of Shandong Provincial Hospital affiliated to Shandong First Medical University (Jinan, China) and complied with all relevant ethical regulations of the Declaration of Helsinki.

*Tumor response and survival analysis.* For the evaluation of tumor response, CT scans were reviewed by specialized radiologists. Tumor assessment was performed at baseline and every 2 cycles according to the RECIST 1.1. On the basis of the best overall response, patients with complete or partial response were considered responders, while others with stable or progressive disease were considered non-responders. The progression-free survival (PFS) and OS were defined as the interval from treatment initiation to the date of clinical progression or death, and to the date of death from any cause, respectively (31). Survival status was obtained through medical records or telephone follow-up every 2 cycles of chemo-immunotherapy. Patients who had not progressed or succumbed to the disease were censored for PFS and OS at last follow-up (August 31, 2021).

*IHC staining and assessment.* All of the cases had available formalin-fixed paraffin-embedded (FFPE) specimens of primary tumors which were obtained from the most recent biopsy before treatment. Tissue sections (4-$\mu$m thick) from each FFPE block were used for FXR, PD-L1 and CD8 IHC staining as previously described (32). Slides were incubated overnight at 4˚C with the following primary antibodies: Anti-bile acid receptor NR1H4 antibody (1:100; product code ab187735), anti-PD-L1 antibody (1:500; product code ab205921) and anti-CD8α antibody (1:100; product code ab101500; all from Abcam). Isotype controls were conducted simultaneously using concentration-matched non-specific mouse or rabbit IgG (product codes ab37355 and ab172730, respectively; both from Abcam). All stained slides were scanned using

a high-resolution digital slide scanner (TissueFAXS plus; TissueGnostics Ltd.) up to a magnification of x200.

For FXR and PD-L1, staining intensity was classified as negative (0), weak (1), moderate (2) and intense (3) according to the degree of dyeing. As for the percentage of stained cells, 0% (0), 1 to 25% (1), 26 to 50% (2), 51 to 75% (3) and 76 to 100% (4) was defined. The IHC score was generated by multiplying the staining intensity and percentage (27). If the staining intensity in a section was diverse, the highest was selected from the scores of different intensities and ratios. Since the median IHC score of FXR and PD-L1 was 6 and 4, respectively, an IHC score of 5 was used as the cut-off value to discriminate low and high expression of FXR and PD-L1, to ensure that the number of NSCLC patients with FXR or PD-L1 low-expression was generally equal to the number of NSCLC patients with FXR or PD-L1 high-expression. For CD8, 4-6 independent high-power fields (HPFs; x200) which represented the densest lymphocytic infiltrates were selected to reflect the extent of $CD8^+$ T-cell infiltration. The average $CD8^+$ T-cell density (cells/HPF) was calculated as the mean value of the 4-6 areas (33). Two proficient pathologists who were blinded to the clinical data independently evaluated the IHC results and reached a final consensus.

*Statistical analysis*. Shapiro-Wilk method was used to assess the normality of the quantitative data. Comparisons between skewed distribution data were performed using Mann-Whitney U test. Categorical variables were compared using chi-square tests or Fisher's exact test. Spearman's rank correlation test was used to assess the correlation between FXR and PD-L1 and between FXR and CD8 in NSCLC. The comparison of infiltrating $CD8^+$ T cells in four groups, according to FXR staining intensity, was performed using Kruskal-Wallis (K-W) rank sum test. Survival curves were estimated by Kaplan-Meier analysis and the log-rank test was utilized to examine the differences between groups. In addition, prognostic factors were evaluated based on univariate and multivariate Cox regression analyses. The variables with univariate regression $P<0.1$ were included in multivariate regression analysis. Statistical analyses were performed using the statistical software SPSS Statistics 26.0 (IBM Corp.) and GraphPad Prism 8.0 (GraphPad Software, Inc.). All tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Baseline characteristics of patients*. A total of 149 patients treated with anti-PD-1-based chemo-immunotherapy were eventually enrolled in the present study. Their clinical and pathological characteristics are listed in Table I. The cohort included 119 men and 30 women with a higher proportion of elderly, and most of the patients were smokers. The majority of these NSCLC cases were non-squamous cell carcinoma (93/149, 62.4%), of which 90 were adenocarcinoma, 2 were sarcomatoid carcinoma and 1 was large cell neuroendocrine carcinoma. More than half of the patients (103/149, 69.1%) were in stage IV at the beginning of anti-PD-1-based chemo-immunotherapy. In the present cohort, all the patients received PD-1 inhibitors combined with chemotherapy as the first-line or higher lines with refractory progression after chemotherapy, radiation or targeted therapy. Among them, the most widely used PD-1 inhibitor was camrelizumab (111/149, 74.5%). There were 40 patients who had oncogene mutations among 67 patients with available gene analysis data. A total of 78 patients (52.3%) and 54 patients (36.2%) were classified as high FXR and PD-L1 expression, respectively (Table I).

*Associations between FXR, PD-L1 expression and response to anti-PD-1-based chemo-immunotherapy in all patients*. As visualized using IHC, the expression of FXR was mainly localized in the nucleus and cytoplasm, while PD-L1 was expressed on the membrane of tumor cells (Fig. 1A). A total of 46 patients were classified as responders according to the RECIST 1.1, and the objective response rate (ORR) to chemo-immunotherapy in the present study was 30.9%. It was revealed that responsive tumors expressed higher levels of both FXR and PD-L1 compared with those irresponsive to chemo-immunotherapy ($P=0.01$ and 0.003, respectively; Fig. 1B and C). Meanwhile, the chi-square test revealed that high FXR and PD-L1 expression levels were associated with a higher ORR in all patients ($P=0.036$ and 0.002, respectively; Fig. 1D and E). These findings underlined the utility of high expression of FXR as a predictive biomarker for immunotherapy in addition to PD-L1.

*Prognostic significance of FXR, PD-L1 and clinicopathological parameters in all patients*. As illustrated in Fig. 2A and B, the Kaplan-Meier and log-rank tests demonstrated that responders to anti-PD-1-based chemo-immunotherapy had both significantly longer PFS and OS than non-responders ($P<0.001$ and $P=0.023$, respectively). There was a non-significant trend towards improved PFS and OS in patients with high FXR expression as compared with their FXR low counterparts (Fig. 2C and D). In addition, PD-L1 high-expression patients were found to have a significantly longer PFS ($P=0.031$; Fig. 2E), as compared with PD-L1 low-expression patients; however, there was no statistical association between PD-L1 expression and OS (Fig. 2F).

To study the prognostic role of FXR and PD-L1 in all patients, a Cox regression model was applied including several clinical characteristics (age, sex, smoking history, histologic type, TNM stage, ECOG PS, lines of therapy and gene mutation state). Given that the number of NSCLC patients with an ECOG PS score of 2 was quite small (4/149, 2.7%), the ECOG PS data were analyzed as a binary variable (0 vs. ≥1). The univariate analysis revealed that TNM stage, line of therapy and PD-L1 expression were associated to PFS (P-values <0.1; Table SI). In multivariate analysis, TNM stage [$P=0.011$; hazard ratio (HR), 2.146; 95% confidence interval (CI), 1.188-3.874)] remained an independent prognostic indicator for PFS. Conversely, only age was defined as an independent prognostic factor for OS ($P=0.021$; HR, 4.117; 95% CI, 1.235-13.727; Table SII). Collectively, neither FXR nor PD-L1 expression could stratify PFS and OS in the present cohort.

*Subgroup analysis of tumor responses and prognosis based on the correlation between FXR and PD-L1*. It was previously reported that $FXR^{high}PD\text{-}L1^{low}$ mouse LLC tumors exhibited an increased susceptibility to PD-1 blockade compared with mock LLC tumors (28). To further investigate whether FXR could be

Table I. Baseline clinical characteristics according to FXR and PD-L1 protein expression of patients with NSCLC in the present cohort.

| | | Expression level of FXR | | | Expression level of PD-L1 | | |
|---|---|---|---|---|---|---|---|
| Variables | All patients no. (%) | Low (%) | High (%) | P-value | Low (%) | High (%) | P-value |
| N | 149 | 71 (47.7) | 78 (52.3) | | 95 (63.8) | 54 (36.2) | |
| Age, years | | | | 0.728[a] | | | 0.467[a] |
| <60 | 44 (29.5) | 20 (45.5) | 24 (54.5) | | 30 (68.2) | 14 (31.8) | |
| ≥60 | 105 (70.5) | 51 (48.6) | 54 (51.4) | | 65 (61.9) | 40 (38.1) | |
| Sex | | | | 0.348[a] | | | 0.711[a] |
| Male | 119 (79.9) | 59 (49.6) | 60 (50.4) | | 75 (63) | 44 (37) | |
| Female | 30 (20.1) | 12 (40.0) | 18 (60.0) | | 20 (66.7) | 10 (33.3) | |
| Smoking history | | | | 0.844[a] | | | 0.275[a] |
| No | 41 (27.5) | 19 (46.3) | 22 (53.7) | | 29 (70.7) | 12 (29.3) | |
| Yes | 108 (72.5) | 52 (48.1) | 56 (51.9) | | 66 (61.1) | 42 (38.9) | |
| Histology | | | | 0.656[a] | | | 0.804[a] |
| Squamous | 56 (37.6) | 28 (50.0) | 28 (50.0) | | 35 (62.5) | 21 (37.5) | |
| Non-squamous | 93 (62.4) | 43 (46.2) | 50 (53.8) | | 60 (64.5) | 33 (35.5) | |
| TNM stage | | | | 0.300[a] | | | 0.110[a] |
| III | 46 (30.9) | 19 (41.3) | 27 (58.7) | | 25 (54.3) | 21 (45.7) | |
| IV | 103 (69.1) | 52 (50.5) | 51 (49.5) | | 70 (68.0) | 33 (32.0) | |
| ECOG PS | | | | 0.985[a] | | | 0.874[a] |
| 0 | 23 (15.4) | 11 (47.8) | 12 (52.2) | | 15 (65.2) | 8 (34.8) | |
| ≥1 | 126 (84.6) | 60 (47.6) | 66 (52.4) | | 80 (63.5) | 46 (36.5) | |
| Therapy line | | | | 0.453[a] | | | 0.014[a] |
| 1st | 94 (63.1) | 47 (50.0) | 47 (50.0) | | 53 (56.4) | 41 (43.6) | |
| ≥2nd | 55 (36.9) | 24 (43.6) | 31 (56.4) | | 42 (76.4) | 13 (23.6) | |
| PD-1 inhibitors | | | | 0.562[a] | | | 0.794[a] |
| Camrelizumab | 111 (74.5) | 53 (47.7) | 58 (52.3) | | 70 (63.1) | 41 (36.9) | |
| Tislelizumab | 14 (9.4) | 5 (35.7) | 9 (64.3) | | 9 (64.3) | 5 (35.7) | |
| Sintilimab | 22 (14.8) | 12 (54.5) | 10 (45.5) | | 15 (68.2) | 7 (31.8) | |
| Pembrolizumab | 1 (0.7) | 1 (100) | 0 (0) | | 0 (0) | 1 (100) | |
| Toripalimab | 1 (0.7) | 0 (0) | 1 (100) | | 1 (100) | 0 (0) | |
| Gene mutations | | | | 0.882[b] | | | 0.580[b] |
| EGFR mutation | 24 (16.1) | 11 (45.8) | 13 (54.2) | | 15 (62.5) | 9 (37.5) | |
| KRAS mutation | 10 (6.7) | 7 (70.0) | 3 (30.0) | | 5 (50.0) | 5 (50.0) | |
| BRAF mutation | 2 (1.3) | 1 (50.0) | 1 (50.0) | | 1 (50.0) | 1 (50.0) | |
| HER-2 mutation | 2 (1.3) | 1 (50.0) | 1 (50.0) | | 2 (100) | 0 (0) | |
| ALK fusion | 1 (0.7) | 0 (0) | 1 (100) | | 0 (0) | 1 (100) | |
| PIK3CA mutation | 1 (0.7) | 0 (0) | 1 (100) | | 1 (100) | 0 (0) | |
| Wild type | 27 (18.1) | 13 (48.1) | 14 (51.9) | | 18 (66.7) | 9 (33.3) | |
| Unknown | 82 (55.0) | 38 (46.3) | 44 (53.7) | | 53 (64.6) | 29 (35.4) | |

[a]P-values were analyzed using Chi-square test. [b]Data were obtained by Chi-square test with mutant vs. wild classification. FXR, farnesoid X receptor; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer; TNM, tumor-node-metastasis; ECOG PS, Eastern Cooperative Oncology Group performance status; PD-1, programmed death-1.

an effective predictor of clinical response to anti-PD-1-based chemo-immunotherapy among PD-L1$^{low}$ patients with NSCLC, a subgroup analysis of tumor responses and prognosis based on the correlation between FXR and PD-L1 was conducted. Firstly, a significant increase of PD-L1 expression in 'FXR low' tumors was found (P=0.016; Fig. 3A), which was consistent with a previous study (28). The chi-square test revealed that FXR was inversely associated with PD-L1 expression in

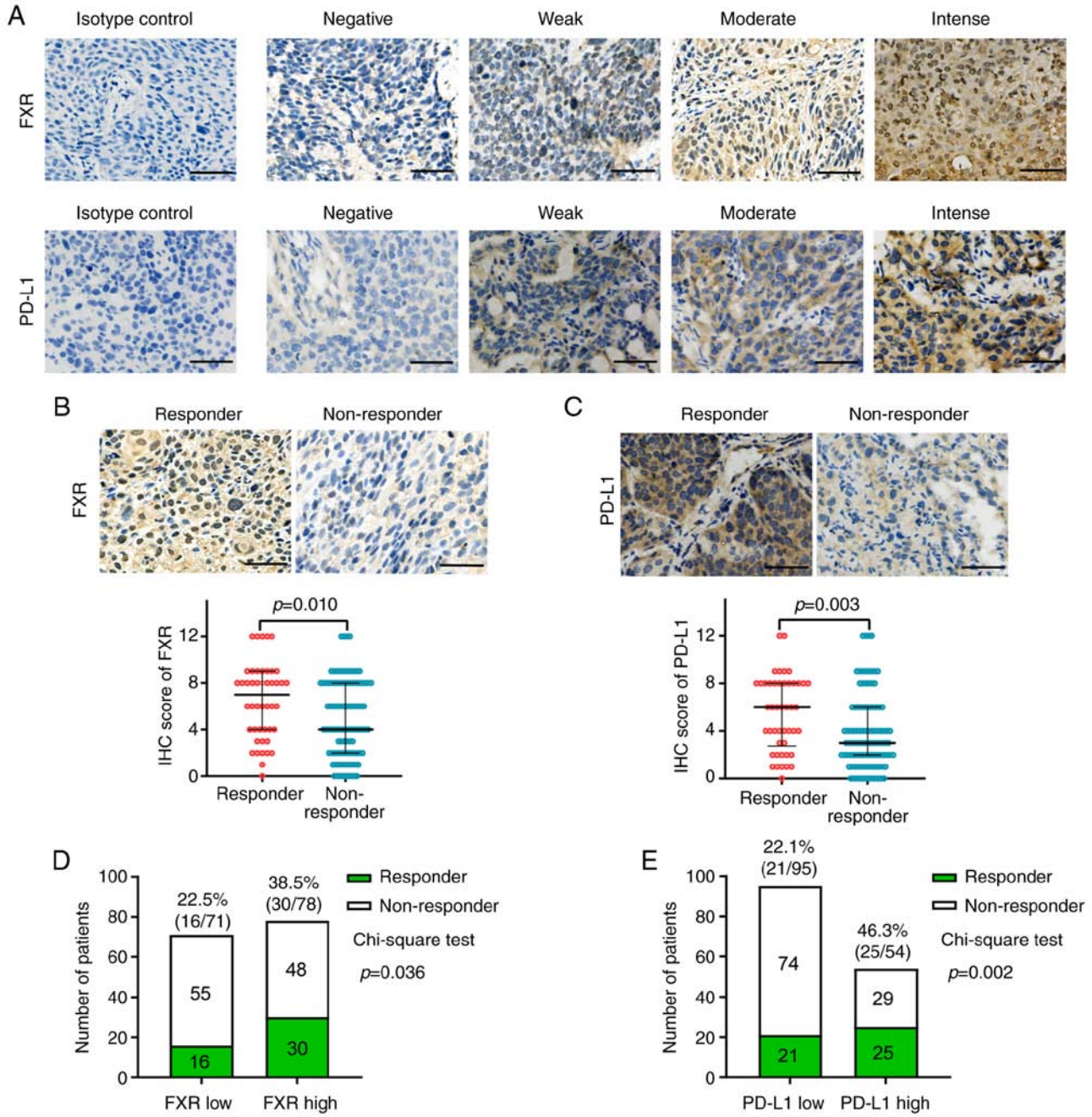


Figure 1. FXR and PD-L1 expression are associated with tumor response to anti-PD-1-based chemo-immunotherapy in patients with NSCLC. (A) Representative IHC images of FXR (upper panel) and PD-L1 (lower panel) expression in NSCLC specimens (Scale bar, 50 $\mu$m). Isotype control: the primary antibody was replaced by nonspecific mouse or rabbit IgG. (B and C) Representative IHC images (upper graphs) and IHC scores (lower graphs) of FXR (B) and PD-L1 (C) in responders vs. non-responders (P=0.01 and P=0.003, respectively; Mann-Whitney U test). Scale bars indicate 50 $\mu$m. Error bars indicate the median and interquartile range. (D) Objective response in patients with low FXR vs. high FXR (ORR 22.5 vs. 38.5%, P=0.036; chi-square test). (E) Objective response in patients with low PD-L1 vs. high PD-L1 (ORR 22.1 vs. 46.3%, P=0.002; chi-square test). The objective response rate (n/N), is shown above each bar. FXR, farnesoid X receptor; PD-L1, programmed death-ligand 1; PD-1, programmed death-1; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry.

specimens with NSCLC (P=0.005; Fig. 3B). In addition, the result of Spearman's correlation analysis demonstrated that there was a significant inverse correlation between FXR and PD-L1 expression in the entire cohort (r=-0.236, P=0.004; Fig. 3C). Furthermore, it was investigated whether PD-L1 expression was different between squamous cell carcinoma and adenocarcinoma in the present study. There was no statistically significant difference in PD-L1 expression between squamous cell carcinoma and adenocarcinoma in 149 specimens with NSCLC enrolled in the present study (data not shown).

Then, four subgroups based on the IHC levels of FXR and PD-L1 were defined (Fig. 4A). Notably, patients with high expression of both PD-L1 and FXR exhibited the highest ORR (60%), followed by the $FXR^{low}PD\text{-}L1^{high}$ group (38.2%). In these PD-L1 high-expression patients, although responsive tumors expressed higher levels of FXR than that of non-responsive tumors (Fig. S1), no significant association was identified between FXR expression and tumor response (Fig. 4A). Of note, patients with high expression of FXR demonstrated a higher ORR as compared with FXR low-expression

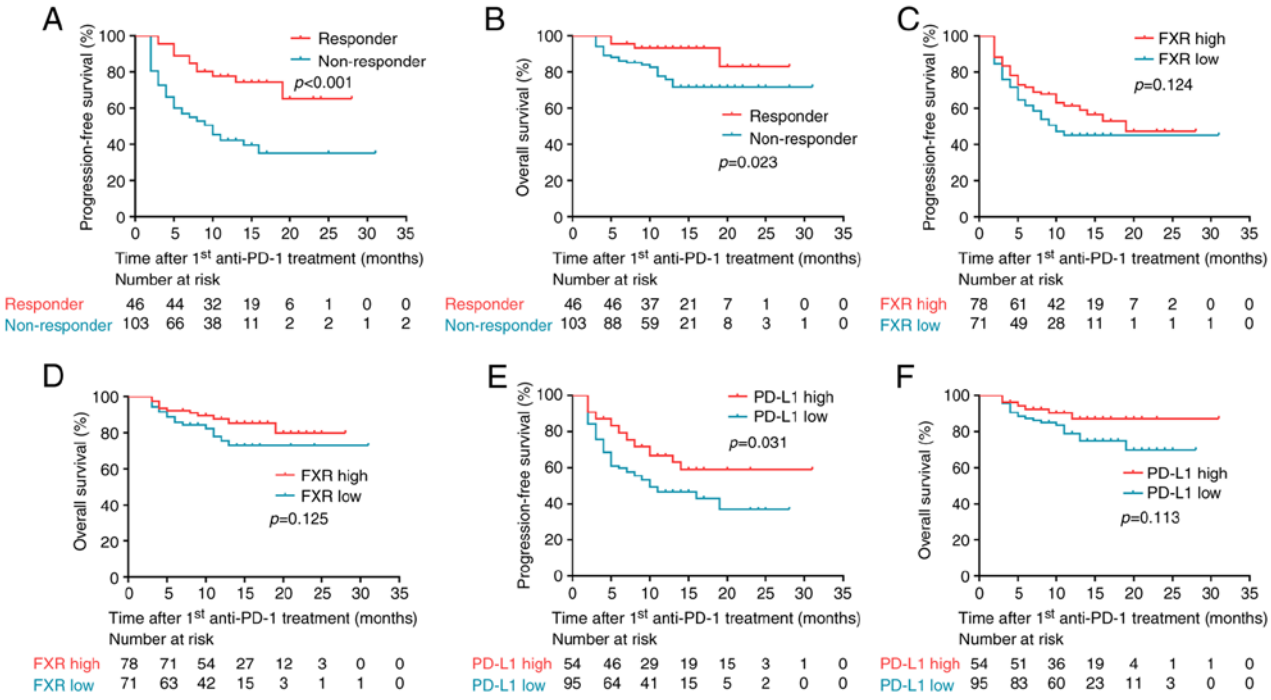


Figure 2. Kaplan-Meier survival curves for PFS and OS of all patients with NSCLC in the present cohort according to tumor response and FXR and PD-L1 expression. (A and B) PFS and OS in responders vs. non-responders (P<0.001 and P=0.023, respectively; log-rank test). (C and D) PFS and OS in patients with low FXR vs. high FXR (log-rank test demonstrated no statistical difference). (E and F) PFS and OS in patients with low PD-L1 vs. high PD-L1 (P=0.031 and P=0.113, respectively; log-rank test). PFS, progression-free survival; OS, overall survival; NSCLC, non-small cell lung cancer; FXR, farnesoid X receptor; PD-L1, programmed death-ligand 1; PD-1, programmed death-1.

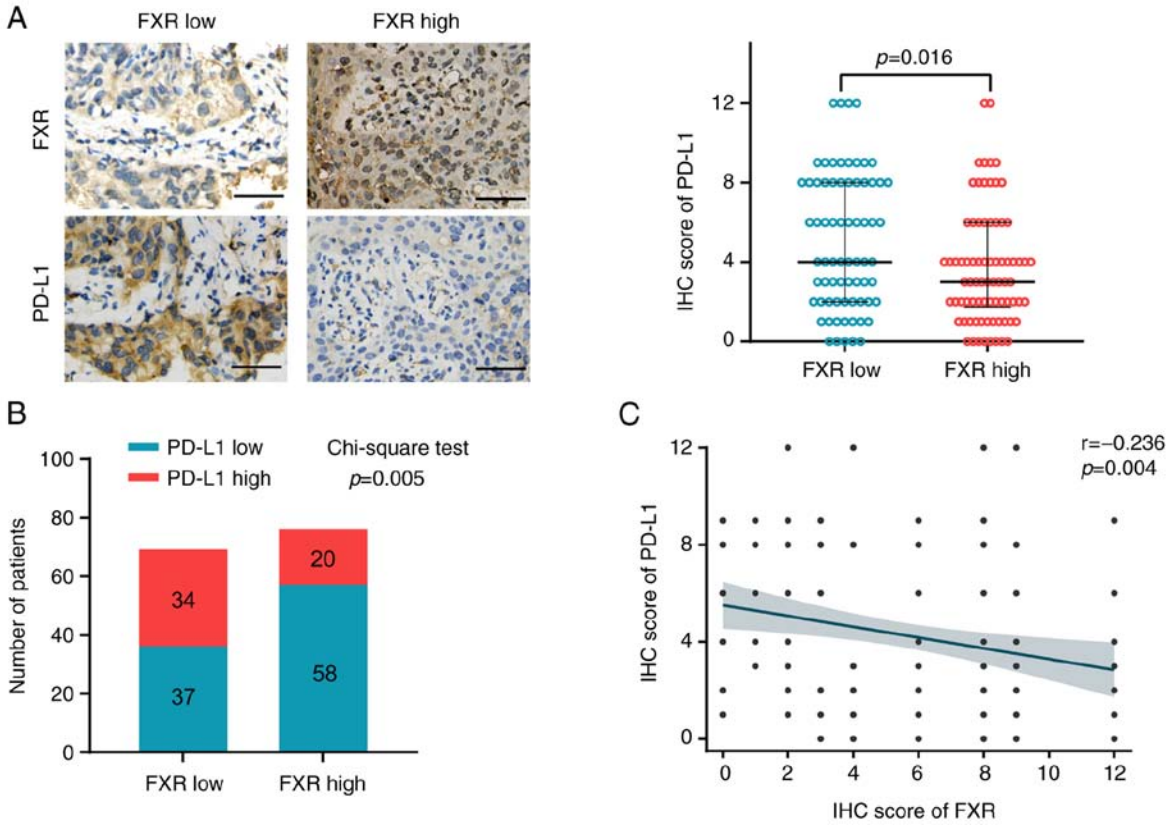


Figure 3. Correlation between FXR and PD-L1 expression in NSCLC specimens. (A) Left panel, representative IHC images of FXR$^{low}$PD-L$^{high}$ and FXR$^{high}$PD-L1$^{low}$ staining in NSCLC specimens. Right panel, the IHC score of PD-L1 in patients with low FXR vs. high FXR (P=0.016, Mann-Whitney U test). Scale bars indicate 50 $\mu$m. Error bars indicate the median and interquartile range. (B) Chi-square test indicated that FXR was inversely associated PD-L1 expression in NSCLC specimens (P=0.005). (C) Spearman's correlation analysis demonstrated a significant inverse correlation between FXR and PD-L1 expression in the entire cohort (r=-0.263, P=0.004). FXR, farnesoid X receptor; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry.

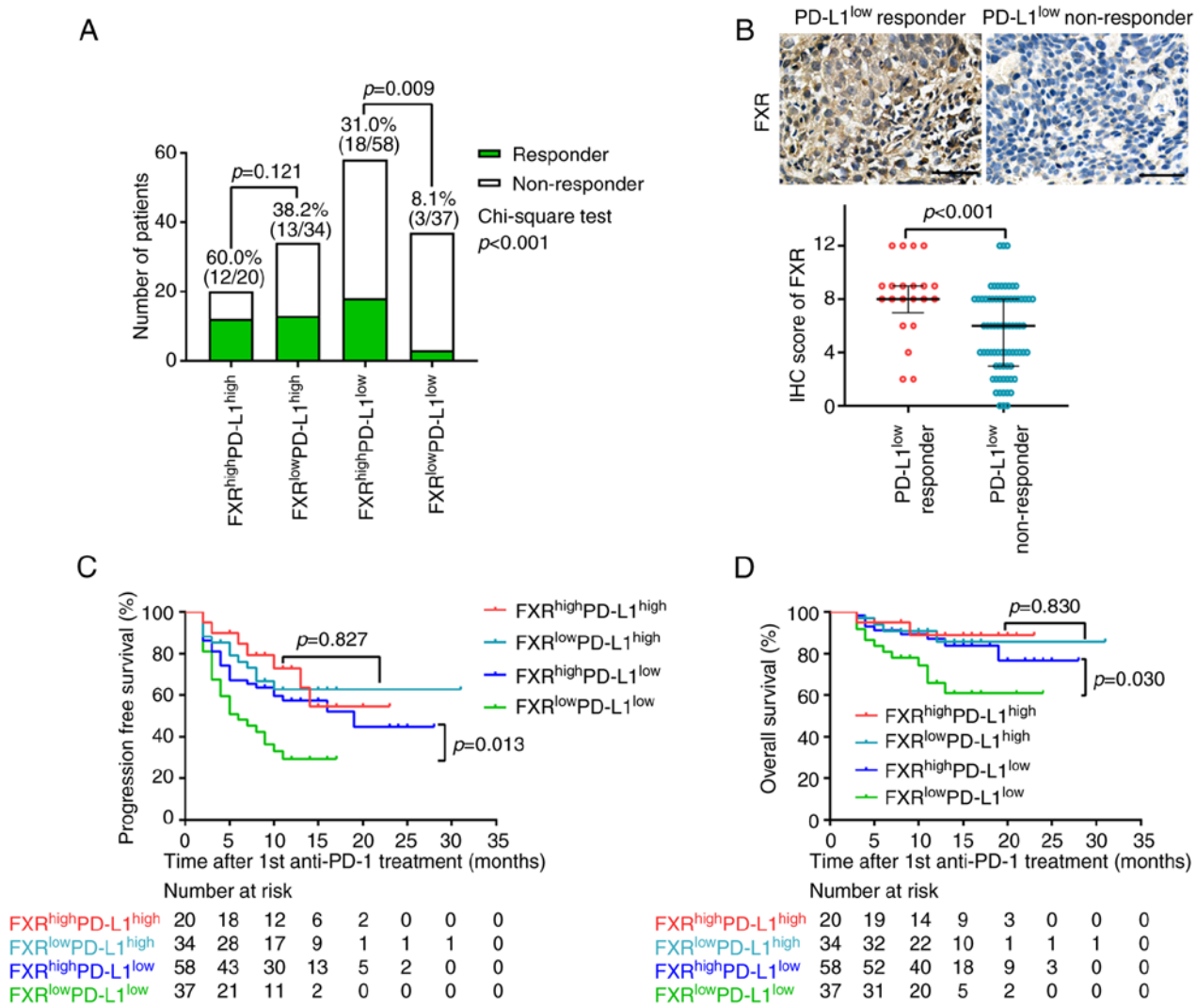


Figure 4. Subgroup analysis of tumor responses and prognosis based on the IHC levels of FXR and PD-L1 in patients with NSCLC receiving anti-PD-1-based chemo-immunotherapy. (A) Objective response in patients with $FXR^{high}PD\text{-}L1^{high}$, $FXR^{low}PD\text{-}L1^{high}$, $FXR^{high}PD\text{-}L1^{low}$, or $FXR^{low}PD\text{-}L1^{low}$ (ORR in $FXR^{high}PD\text{-}L1^{low}$ vs. $FXR^{low}PD\text{-}L1^{low}$ patients, 31 vs. 8.1%, P=0.009; chi-square test). The objective response rate (n/N), is shown above each bar. (B) Representative IHC images (upper panels) and IHC score (lower panel) of FXR in $PD\text{-}L1^{low}$ responders vs. $PD\text{-}L1^{low}$ non-responders (P<0.001; Mann-Whitney U test). Scale bars indicate 50 $\mu$m. Error bars indicate the median and interquartile range. (C and D) Kaplan-Meier survival curves for PFS and OS of four subgroups (PFS and OS in $FXR^{high}PD\text{-}L1^{low}$ vs. $FXR^{low}PD\text{-}L1^{low}$ patients, P=0.013 and P=0.03, respectively; log-rank test). IHC, immunohistochemistry; FXR, farnesoid X receptor; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer; PD-1, programmed death-1; PFS, progression-free survival; OS, overall survival.

patients in the presence of PD-L1 low-expression (31.0 vs. 8.1%, P=0.009). Additionally, $PD\text{-}L1^{low}$-responsive tumors expressed significantly increased FXR compared with the $PD\text{-}L1^{low}$-non-responsive tumors (P<0.001; Fig. 4B). Collectively, these results suggested FXR as a promising predictive biomarker for clinical efficacy to anti-PD-1-based chemo-immunotherapy when PD-L1 is low or negative.

The Kaplan-Meier survival curves in Fig. 4C and D revealed that high FXR expression was associated with both longer PFS (P=0.013) and OS (P=0.03) among the $PD\text{-}L1^{low}$ patients with NSCLC. However, consistent with the association with therapeutic responses, the extent of FXR expression in $PD\text{-}L1^{high}$ patients was not associated with either PFS or OS (Fig. 4C and D).

*Prognostic significance of FXR in $PD\text{-}L1^{low}$ patients*. Cox regression models were then applied, including clinical variables to verify the prognostic value of FXR in $PD\text{-}L1^{low}$ patients. As presented in Table II, TNM stage and FXR expression were found to be significantly correlated with PFS in univariate Cox regression analysis. These two variables were then analyzed in a multivariate Cox regression model. Intriguingly, FXR expression was still identified as an independent predictor for PFS in $PD\text{-}L1^{low}$ patients with NSCLC (P=0.038; HR, 0.552; 95% CI, 0.315-0.967).

As for the univariate Cox regression analysis for OS in $PD\text{-}L1^{low}$ patients, age and FXR expression were found to stratify OS significantly at the level of P<0.1 (Table III). Additionally, multivariate analysis showed that FXR expression remained an independent prognostic indicator for OS in $PD\text{-}L1^{low}$ patients with NSCLC (P=0.029; HR, 0.377; 95% CI, 0.157-0.905).

*Correlation between FXR and infiltrating $CD8^+$ T cells in NSCLC*. The predictive value of FXR on anti-PD-1-based

Table II. Univariate and multivariate cox regression analysis for progression-free survival in PD-L1$^{low}$ patients.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Variables | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Age, years | | | | | | |
| <60 | 1 | | | | | |
| ≥60 | 0.775 | 0.433-1.389 | 0.392 | | | |
| Sex | | | | | | |
| Male | 1 | | | | | |
| Female | 0.847 | 0.424-1.693 | 0.639 | | | |
| Smoking history | | | | | | |
| No | 1 | | | | | |
| Yes | 0.846 | 0.472-1.515 | 0.573 | | | |
| Histology | | | | | | |
| Squamous | 1 | | | | | |
| Non-squamous | 1.076 | 0.603-1.918 | 0.805 | | | |
| TNM stage | | | | | | |
| III | 1 | | | 1 | | |
| IV | 2.174 | 1.052-4.495 | 0.036 | 2.017 | 0.971-4.189 | 0.060 |
| ECOG PS | | | | | | |
| 0 | 1 | | | | | |
| ≥1 | 1.127 | 0.507-2.503 | 0.770 | | | |
| Therapy line | | | | | | |
| 1st | 1 | | | | | |
| ≥2nd | 1.25 | 0.720-2.171 | 0.428 | | | |
| Gene mutations | | | | | | |
| Wild | 1 | | | | | |
| Mutant | 1.725 | 0.728-4.086 | 0.215 | | | |
| Expression level of FXR | | | | | | |
| Low | 1 | | | 1 | | |
| High | 0.515 | 0.295-0.901 | 0.020 | 0.552 | 0.315-0.967 | 0.038 |

The variables with univariate regression P<0.1 were included in multivariate regression analysis. PD-L1, programmed death-ligand 1; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; ECOG PS, Eastern Cooperative Oncology Group performance status; FXR, farnesoid X receptor.

chemo-immunotherapy in the perspective of TME was then sought to be explained. Since CD8$^+$ T cells represent the most crucial tumoricidal effector cells and the main target of the PD-L1/PD-1 checkpoint pathway in the TME (34,35), the infiltration of CD8$^+$ T cells in specimens with NSCLC was examined. Representative microphotographs of the infiltration levels of different CD8$^+$ T cells are shown in Fig. 5A. The cells positively stained for CD8 were semi-quantified and low or high groups were defined according to the median value. IHC evaluation revealed a statistically significant decrease of infiltrating CD8$^+$ T cells in FXR$^{high}$ NSCLC specimens (P=0.014; Fig. 5B). Chi-square analysis demonstrated that FXR expression was inversely associated with the infiltration of CD8$^+$ T cells in specimens with NSCLC (P=0.004; Fig. 5C). Importantly, the Spearman's correlation analysis revealed that there was a significant inverse correlation between FXR expression and infiltrating CD8$^+$ T cells in the enrolled 149 specimens with NSCLC (r=-0.217, P=0.008; Fig. 5D). The CD8 expression data were analyzed in four groups, according to FXR staining intensity (negative, weak, moderate and intense). However, the K-W analysis showed that there was no difference in the degree of infiltration of CD8$^+$ T cells among the four FXR staining groups in the present study (data not shown). Thus, it was considered that the immunosuppressive effects of FXR previously reported (28) in *in vitro* co-culture and mouse models could also be recapitulated in clinical patients with NSCLC.

## Discussion

In the past decade, the emergence of anti-PD-1/PD-L1-directed immunotherapy has significantly changed the clinical management and outcome of patients with advanced NSCLC (4-7). High expression of tumor PD-L1 predicts clinical efficacy of

Table III. Univariate and multivariate cox regression analysis for overall survival in PD-L1$^{low}$ patients.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Variables | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Age, years | | | | | | |
| <60 | 1 | | | 1 | | |
| ≥60 | 2.954 | 0.870-10.035 | 0.083 | 3.149 | 0.925-10.723 | 0.067 |
| Sex | | | | | | |
| Male | 1 | | | | | |
| Female | 0.546 | 0.161-1.858 | 0.333 | | | |
| Smoking history | | | | | | |
| No | 1 | | | | | |
| Yes | 1.555 | 0.568-4.257 | 0.39 | | | |
| Histology | | | | | | |
| Squamous | 1 | | | | | |
| Non-squamous | 0.567 | 0.239-1.343 | 0.197 | | | |
| TNM stage | | | | | | |
| III | 1 | | | | | |
| IV | 1.817 | 0.609-5.422 | 0.284 | | | |
| ECOG PS | | | | | | |
| 0 | 1 | | | | | |
| ≥1 | 1.898 | 0.442-8.157 | 0.389 | | | |
| Therapy line | | | | | | |
| 1st | 1 | | | | | |
| ≥2nd | 0.844 | 0.355-2.006 | 0.701 | | | |
| Gene mutations | | | | | | |
| Wild | 1 | | | | | |
| Mutant | 0.265 | 0.047-1.486 | 0.131 | | | |
| Expression level of FXR | | | | | | |
| Low | 1 | | | 1 | | |
| High | 0.398 | 0.167-0.953 | 0.039 | 0.377 | 0.157-0.905 | 0.029 |

The variables with univariate regression P<0.1 were included in multivariate regression analysis. PD-L1, programmed death-ligand 1; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; ECOG PS, Eastern Cooperative Oncology Group performance status; FXR, farnesoid X receptor.

PD-1/PD-L1 blockade (12,13), meanwhile a few PD-L1$^{low/negative}$ patients still benefit from these drugs (6,19). Thus, there is an urgent need to further stratify patients who can derive benefit from immunotherapy from those who cannot within the PD-L1$^{low/negative}$ group. In the present study, 149 clinical NSCLC specimens were screened for FXR and PD-L1 expression to determine their predictive value for anti-PD-1-based chemo-immunotherapy. The present results showed that high FXR and PD-L1 expression levels were associated with a higher ORR in the entire cohort. The inverse correlation between the expression of FXR and PD-L1 in NSCLC specimens was also verified, consistent with a previous study (28). Notably, subgroup analysis revealed that high FXR expression was associated with a higher ORR, as well as longer PFS and OS among PD-L1$^{low}$ patients with NSCLC. Mechanistically, a statistically significant decrease of infiltrating CD8$^{+}$ T cells in FXR$^{high}$ NSCLC specimens was observed. The present study provided a brand-new stratification, recommending FXR$^{high}$PD-L1$^{low}$ as a potential predictive biomarker for PD-L1$^{low/negative}$ NSCLC patients who can benefit from anti-PD-1-based chemo-immunotherapy.

Previously, emerging evidence supported differential roles for FXR in carcinogenesis. It was previously reported that FXR overexpression contributed to lymphatic metastasis of human pancreatic cancer (36). Another study demonstrated a vital role of FXR in endothelial cell motility and vascular tube formation, essential for tumor angiogenesis (37). It was previously found that FXR promotes NSCLC cell proliferation through increasing *CCND1* transcription (27), and that enforced FXR expression constructs an immunosuppressive microenvironment in mouse LLC tumors (28). The present study provided compelling clinical evidence to extend FXR function as an indicator of sensitivity to immune-related therapies. The data showed that high FXR expression was associated with a higher ORR in patients with NSCLC undergoing anti-PD-1-based

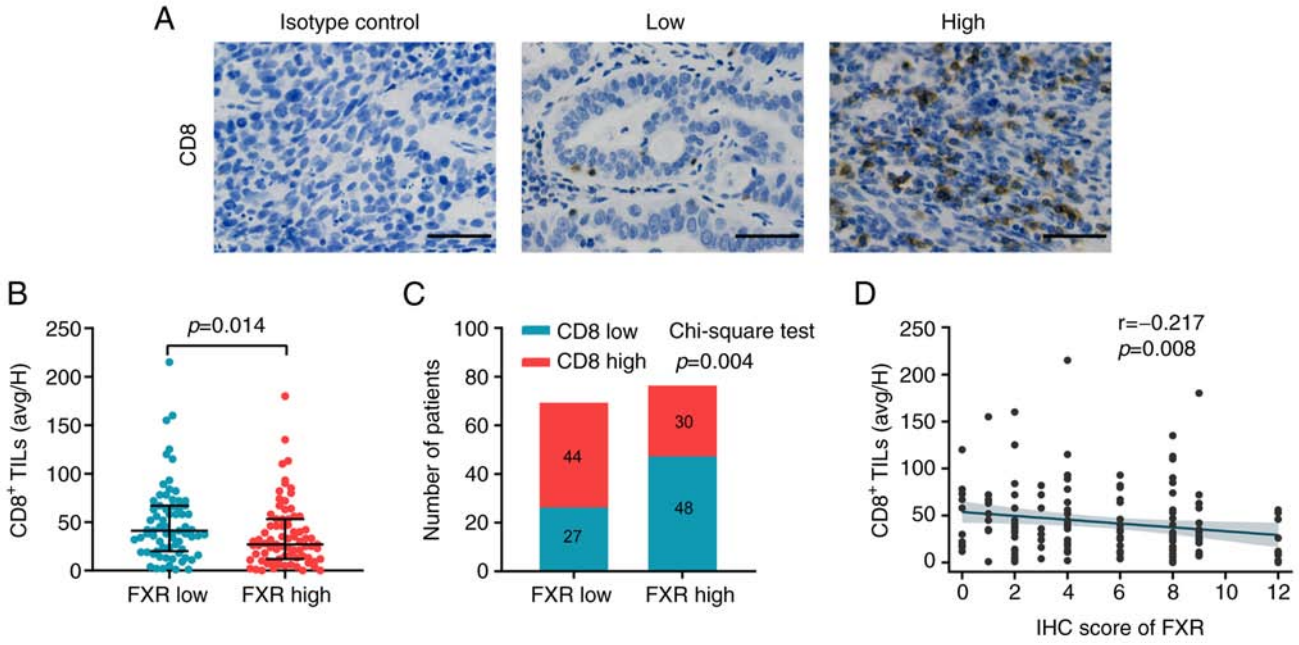


Figure 5. FXR is inversely correlated with CD8 expression in NSCLC specimens. (A) Representative IHC images of low or high CD8 expression in NSCLC specimens (Scale bar, 50 $\mu$m). Isotype control: the primary antibody was replaced by nonspecific rabbit IgG. (B) The number of cells positively stained for CD8 in patients with low FXR vs. high FXR (P=0.014; Mann-Whitney U test). Error bars indicate the median and interquartile range. (C) Chi-square analysis demonstrated that FXR expression was inversely associated with infiltration of CD8$^+$ T cells in NSCLC specimens (P=0.004). (D) Spearman's correlation analysis revealed a significant inverse correlation between FXR expression and infiltrating CD8$^+$ T cells in the 149 NSCLC specimens (r=-0.217, P=0.008). FXR, farnesoid X receptor; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry.

chemo-immunotherapy. Additionally, there was a non-significant trend toward improved PFS and OS in FXR high-expression group as compared with FXR low-expression group with the same treatment. Consistent with the present findings, previous studies have reported that FXR activation enhanced the chemo-sensitivity of biliary tract and colorectal cancer cells to oxaliplatin and cisplatin, respectively (38,39).

PD-L1 is currently approved as a predictive biomarker for anti-PD-1/PD-L1 response in cancer treatment, including NSCLC (11,40). However, the predictive value of tumoral PD-L1 is discordant since in clinical trials it was identified that a proportion of PD-L1$^{low/negative}$ patients can also derive clinical benefit from PD-1/PD-L1 blockade (6,19). In the present study, 30.9% (46/149) of the patients were classified as responders to chemo-immunotherapy, which is consistent with the ORR reported by Carbone *et al* (41) in stage IV or recurrent patients with NSCLC treated with the combination of nivolumab and platinum doublet chemotherapy. Interestingly, it was observed that 22.1% (21/95) of the patients with low PD-L1 expression responded to anti-PD-1-based chemo-immunotherapy, consistent with a previous study which revealed that the anti-PD-L1 antibody MPDL3280A resulted in an ORR of 20% in PD-L1 low-expression patients with NSCLC (42). Possible explanations for this discordance may include the fact that PD-L1 expression is dynamic and heterogeneous, both within the same tumor and between primary and metastatic lesions in the same patient (16). An alternative explanation could rely on the different testing platforms and different cut-off values for PD-L1 positivity (17,18). There are currently no approved predictors that can guide treatment decision for the PD-L1$^{low/negative}$ patients. In the present study, the inverse correlation between FXR and PD-L1 expression in NSCLC specimens was verified. Consistently, a previous study demonstrated that FXR can suppress PD-L1 transcription by binding to the putative FXR element in *PD-L1* promoter. In addition, SHP, a downstream target gene of FXR, and EGFR signals are also involved in FXR-induced PD-L1 downregulation in NSCLC cells (28). Notably, stratifying by FXR and PD-L1 expression showed that FXR$^{high}$PD-L1$^{low}$ patients with NSCLC displayed a significantly higher ORR, as well as longer PFS and OS, compared with FXR$^{low}$PD-L1$^{low}$ patients among the PD-L1$^{low}$ group. High FXR expression was established to be an independent predictor for PFS and OS in PD-L1$^{low}$ patients with NSCLC receiving anti-PD-1-based chemo-immunotherapy. In line with the present findings, baseline serum IL-6 level was demonstrated to be a potential biomarker for predicting the efficacy and survival outcome of PD-1/PD-L1 inhibitors, even in PD-L1$^{low/negative}$ patients with NSCLC (43). Similarly, SWI/SNF chromatin remodeling gene alterations were positively associated with objective responses in immune checkpoint inhibitor-treated advanced pancreatic cancer in the presence of low PD-L1 expression (44). Based on the encouraging results, combining FXR with PD-L1 IHC testing could be considered to identify NSCLC subsets with high likelihood of deriving benefit from immune-related therapies.

Finally, the underlying mechanisms of chemo-immunotherapy responsiveness in FXR high-expression patients with NSCLC in the perspective of the TME was investigated. It is well-known that the tumor-infiltrating CD8$^+$ T cells represent the most crucial tumoricidal effector cells, as well as the main target of the PD-L1/PD-1 checkpoint pathway (34,35). In accordance with a previous study (28), a statistically significant decrease of infiltrating CD8$^+$ T cells in the more responding FXR$^{high}$ NSCLC was observed.

There was a significantly inverse correlation between FXR and CD8 expression in NSCLC specimens, suggesting that the restrained tumor-infiltrating $CD8^+$ T cells, rather than the fully activated ones, are more readily to be rescued by anti-PD-1 in FXE high-expression tumors. This theory is supported by a previous study, which revealed that objective response to PD-1/PD-L1 blockade mainly occurs in tumors with adaptive immune-resistant infiltrating T cells (45). The present study cannot exclude the possibility that other immune cell populations also contribute to the increased responsiveness to anti-PD-1-based chemo-immunotherapy in FXR high-expression NSCLC. Future studies are required to elucidate the interplay between tumoral FXR expression and other tumor-infiltrating immune cells in the TME.

There are certain limitations in the present study. First, this is a retrospective, single-center study, and the sample size is relatively small to perform an elaborate statistical analysis. Large-scale prospective multi-center studies could be helpful to validate the present findings. Secondly, the OS data were slightly immature since the majority of patients did not reach the primary endpoint of death, which may limit the prognostic value of OS. Third, the molecular basis by which FXR suppresses tumor-infiltrating $CD8^+$ T cells in NSCLC remains to be further explored. In FXR high-expression patients with NSCLC, the majority (58/78, 74.4%) were classified as $FXR^{high}PD\text{-}L1^{low}$, while the minority (20/78, 25.6%) were classified as $FXR^{high}PD\text{-}L1^{high}$, which can be explained by the fact that FXR suppresses PD-L1 expression in NSCLC (28). However, in FXR low-expression patients with NSCLC, $FXR^{low}PD\text{-}L1^{low}$ accounted for 52.1% (37/71) and $FXR^{low}PD\text{-}L1^{high}$ accounted for 47.9% (34/71), which indicated that the expression of PD-L1 may be regulated by other factors in FXR low-expression NSCLC, beyond the scope of this manuscript. Despite these limitations, this represents the first study investigating the predictive value of FXR expression in cancer immunotherapy.

In conclusion, it was reported that high FXR and PD-L1 expression levels were associated with higher ORR in patients with NSCLC. It is noteworthy that FXR was inversely correlated with PD-L1 expression in specimens with NSCLC, and that $FXR^{high}PD\text{-}L1^{low}$ phenotype was associated with a higher ORR, as well as longer PFS and OS among the $PD\text{-}L1^{low}$ group. Mechanistic insights revealing that the infiltrating $CD8^+$ T cells were significantly decreased in $FXR^{high}$ NSCLC tumors were also provided. The present study recommended the $FXR^{high}PD\text{-}L1^{low}$ signature as a promising predictor of response to anti-PD-1-based chemo-immunotherapy in $PD\text{-}L1^{low/negative}$ NSCLC, providing clinical evidence for the development of complementary biomarkers for immune-related therapies.

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## Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

WY and SJ designed and conceived the study. LW and XX collated and analyzed the clinicopathological data and wrote the manuscript. BS and JS performed the IHC experiments. BL and XW performed the statistical analysis and data interpretation. WY and SJ reviewed and revised the manuscript and confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The study protocol was approved (approval no. NSFC-2019-05) by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong First Medical University (Jinan, China) and complied with the Helsinki declaration and the approved guidelines of our institution. Written informed consent was obtained from all participants.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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